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(54) Title: SIRNA MEDIATED GENE SILENCING IN TRANSGENIC ANIMALS

(57) Abstract: The present invention relates to a process that enables constitutive and inducible gene knock down in living organisms using a short hairpin RNA expression vector integrated into the genome of the organism.

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SiRNA Mediated Gene Silencing in Transgenic Animals

The present invention relates to a process that enables constitutive and inducible gene knock down in living organisms using a short hairpin RNA expression vector integrated into the genome of the organism.

Background of the Invention

RNA interference (RNAi) has been discovered some years ago as a tool for inhibition of gene expression (Fire, A. et al., Nature 391, 806-811 (1998)). It based on the introduction of double stranded RNA (dsRNA) molecules into cells, whereby one strand is complementary to the coding region of a target gene. Through pairing of the specific mRNA with the introduced RNA molecule, the mRNA is degraded by a cellular mechanism. Since long dsRNA provokes an interferon response in mammalian cells, the technology was initially restricted to organisms or cells showing no interferon response (Bass, B.L., Nature 411, 428-429 (2001)). The finding that *short* (<30 bp) *interfering RNAs* (siRNA) circumvent the interferon response extended the application to mammalian cells (Elbashir, S.M. et al., Nature 411, 494-498 (2001)).

Although RNAi in mice has been in principle demonstrated, the current technology does not allow performing systematic gene function analysis *in vivo*. So far the inhibition of gene expression has been achieved by injection of *purified* siRNA into the tail vain of mice (McCaffrey, A.P. et al., Nature 418, 38-39 (2002); Lewis, D.H. et al., Nature Genet. 32, 107-108 (2002)). Using this approach, gene inhibition is restricted to specific organs and persists only a few days. A further improvement of the siRNA technology is based on the intracellular transcription of short hairpin RNA (shRNA) molecules using gene expression vectors (see Fig. 1; Brummelkamp, T.R. et al., Science 296, 550-553 (2002); Paddison, P.J. et al, Genes Dev. 16, 948-958 (2002); Yu, J.Y. et al., Proc. Natl. Acad. Sci. USA 99, 6047-6052 (2002); Sui, G. et al., Proc. Natl. Acad. Sci. USA 99, 5515-5520 (2002); Paul, C.P. et al., Nature Biotechnol. 20, 505-508 (2002); Xia, H. et al., Nat. Biotechnol. 10, 1006-10 (2002); Jacque,

J.M. et al., Nature 418(6896):435-8 (2002)). The activity of shRNA in mice has been demonstrated by McCaffrey et al., 2002 through injection of shRNA expression vectors into the tail vain. Again, gene inhibition was temporally and spatially restricted. Although these results demonstrate that the mechanism of shRNA mediated gene silencing is functional in mice, they do not clarify whether constitutive RNAi can be achieved in transgenic animals. Brummelkamp, T.R. et al., Science 296, 550-553 (2002), Paddison, P.J. et al., Genes Dev. 16, 948-958 (2002), Hemann, M.T. et al., Nat. Genet. 33(3):396-400 (2003); and Devroe, E. et al., BMC Biotechnol. 2(1):15 (2002) have shown the long-term inhibition of gene expression through stable integration of shRNA vectors in cultivated cell lines. These experiments included random integration of shRNA transgenes and screening for clones with appropriate siRNA expression, which is not applicable for testing of a large number of different shRNA transgenes in mice. Finally, several reports have demonstrated shRNA-mediated gene silencing in transgenic mice and rats (Hasuwa, H. et al., FEBS Lett. 532(1-2):227-30 (2002); Carmell, M.A. et al., Nat. Struct. Biol. 10(2):91-2 (2003); Rubinson, D.A. et al., Nat. Genet. 33(3):401-6 (2003); Kunath, T. et al., Nat. Biotechnol. (Apr. 7 2003)). However, these experiments again included random integration of shRNA transgenes resulting in variable levels and patterns of shRNA expression. Thus, testing of ES cell clones or mouse lines with appropriate shRNA expression had been required, which is a laborious and time-consuming undertaking.

The *in vivo* validation of genes by RNAi mediated gene repression in a large scale setting requires the expression of siRNA at sufficiently high levels and with a predictable pattern in multiple organs. Targeted transgenesis provides the only approach to achieve reproducible expression of transgenes in the living organism (e.g. mammalians such as mice). It has been, however, questionable whether a single copy of a siRNA expression vector integrated into the genome would result in sufficiently high levels of siRNA required for RNAi-mediated gene inhibition in multiple organs of the living organism.

Most siRNA expression vectors are based on polymerase III dependent (Pol III) promoters (U6 or H1) that allow the production of transcripts carrying

only a few non-homologous bases at their 3' ends. It has been shown that the presence of non-homologous RNA at the ends of the shRNA stretches lower the efficiency of RNAi mediated gene silencing (Xia et al., Nat. Biotechnol. 10, 1006-10 (2002)). However, it has been unpredictable for a person skilled in the art which genomic region within the living organism promotes an appropriate expression pattern of the Pol III promoter driven shRNA constructs required for RNAi-mediated gene inhibition in multiple organs of the living organism.

For the temporal control of RNAi mediated gene silencing in transgenic cells lines and living organism, a tight system for inducible siRNA expression is needed. Inducible gene expression systems based on the tetracycline dependent repressor are known. It is, however, not obvious whether a stably integrated, single copy configuration of these systems can be created that allows inducible RNAi in multiple organs without background activity.

Summary of the Invention

It has now been found that the modification of murine embryonic stem (ES) cells by the introduction of shRNA expression vectors through targeted transgenesis allows the inhibition of gene expression in mice generated from these modified ES cells. The invention thus provides

(1) the use of an expression vector comprising a short hairpin RNA (shRNA) construct under control of a ubiquitous promoter for preparing an agent for constitutive and/or inducible gene knock down (hereinafter also referred to as "RNAi mediated inhibition of gene expression") in a living organism, in tissue culture or in cells of a cell culture;

(2) a method for constitutive and/or inducible gene knock down in a living organism, in tissue culture or in cells of a cell culture which comprises stably integrating an expression vector as defined in (1) above into the genome of the living organism, in the genome of the tissue culture or into the genome of the cells of the cell culture;

(3) a living organism, tissue culture or cell culture having stably integrated, preferably at a polymerase II dependent locus of the living organism, tissue

culture or cells of the cell culture, an expression vector as defined in (1) above; and

(4) an expression vector as defined in (1) above.

The agent of embodiment (1) is suitable for *in vivo* and *in vitro* applications.

The living organisms in embodiments (1) to (3) above are preferably vertebrates. In embodiments (2) and (3) the living organisms are more preferably non-human vertebrates. The tissue cultures and the cells of the cell cultures of (1) to (3) above are preferably derived from vertebrates. The method of embodiment (2) includes *in vivo* and *in vitro* reaction schemes.

Short Description of the Figures

Fig. 1: Mechanism of RNAi through shRNA expression. A: expression of shRNA. B: shRNA processing. C: Pairing of siRNA with complementary mRNA strands. D: Cleavage of mRNA.

Fig 2: Pol III dependent shRNA expression vector (constitutive). Insertion of an shRNA expression vector into a ubiquitously expressed genomic locus. Transcription through the upstream Pol II dependent promoter will be stopped by a synthetic polyadenylation signal (pA) and the hGH pA. The Pol III dependent promoter controls the expression of shRNA. The transcript is stopped by five thymidine bases. The exact sequence of suitable target vectors is given in SEQ ID Nos:6 and 8.

Fig. 3: Tet repression system. tTS is a doxycycline controlled transcriptional silencer consisting of the tet repressor and the KRAB-AB domain of the Kid-1 protein. This fusion protein binds to the pol III dependent promoter via the tet operator sequences only in the absence of doxycycline and represses transcription.

Fig. 4: Vectors for Pol III dependent promoter based tet-repression system (inducible). Insertion of a shRNA expression vector into a ubiquitous expressed genomic locus. The transcription of the Pol II dependent promoter will be stopped by the synthetic polyadenylation signal (pA) and a hGH pA. An

inducible Pol III dependent promoter controls the expression of shRNA. The transcript is stopped by five thymidine bases.

Fig. 5: Principle of the Doxycycline inducible gene expression system. rtTA2^{M2} is a doxycycline-controlled transcriptional activator consisting of the reverse tet repressor and the VP16 activation domain of the Herpes simplex virus VP16 protein. This fusion protein binds to a minimal CMV-promoter via the tet operator sequences in the presence of doxycycline and activates transcription. tTS functions in a similar fashion as explained in Fig. 3.

Fig. 6: Vectors for Pol II dependent promoter based tet-system (inducible). Insertion of a shRNA expression vector into a ubiquitous expressed genomic locus. The rtTA2^{M2} is expressed under the control of the ubiquitous Pol II dependent promoter, stopped by a synthetic polyadenylation signal (pA) and a hGH pA. An inducible Pol II dependent promoter controls the expression of shRNA. The transcript is stopped by a short polyadenylation signal.

Figure 7: ShRNA-mediated inhibition of luciferase gene expression in embryonic stem cells. All cells carried stably integrated expression cassettes of firefly- (Fluc) and Renilla luciferases (Rluc). Simultaneous expression of a Fluc-specific shRNA under control of the H1- (H1-shRNA) or U6-promoter (U6-shRNA) resulted in a ~75% and ~60% reduction of firefly luciferase activity, respectively. The values of Fluc activity were normalized by using the Rluc activity for reference.

Fig. 8: Vectors for a U6 promoter based tet-repression system (inducible). Transcription of the Pol II promoter will be stopped by the synthetic polyadenylation signal (pA) and a hGH pA sequence. The inducible U6 promoter controls expression of the Fluc-specific shRNA. Transcription of the shRNA is stopped by five thymidine bases. TetR is a doxycycline controlled repressor (Gossen and Bujard, PNAS. 89: 5547-5551 (1992)) expressed by the CAGGS-promoter. The tetR binds to the pol III promoter via the tet operator sequences only in the absence of doxycycline and blocks transcription.

Fig. 9: Vectors for H1 promoter based tet-repression system (inducible). The inducible H1 promoter controls the expression of shRNA. TetR is a doxycycline

controlled repressor expressed by the CAGGS-promoter. The tetR binds to the pol III promoter via the tet operator sequences only in the absence of doxycycline and blocks transcription.

Figure 10: Inducible RNA interference in embryonic stem cells using tetracycline dependent U6 promoters. All cells carried stably integrated expression cassettes of firefly luciferase (Fluc), Renilla luciferase (Rluc), and the tet repressor. Doxycycline-inducible expression of a Fluc-specific shRNA under control of the $U6_{tetO1}$- (Art4.12 RNAi 23/36 C2) or $U6_{tetO2}$-promoter (Art4.12 RNAi 24/36 A-D11) resulted in a ~30% and ~50% reduction of firefly luciferase activity, respectively. The values of Fluc activity were normalized by using the Rluc activity for reference (figure 12).

Figure 11: Inducible RNA interference using tetracycline dependent H1 promoters NIH3T3. Cells were transiently transfected with firefly luciferase (Fluc), tet repressor, LacZ, and shRNA expression vectors. Doxycycline-inducible expression of the Fluc-specific shRNA under control of the $H1_{tetO2/5'}$-, $H1_{tetO2/3'}$-, or $H1_{tetO2/5'+3'}$-promoter resulted in a ~40%, ~80%, and ~40% reduction of firefly luciferase activity, respectively. The values of Fluc activity were normalized by using the LacZ activity for reference.

Figure 12: Experimental strategy. Genes of the firefly and Renilla luciferases are expressed through the endogenous rosa26 promoter via splice acceptor sites. Specific shRNA mediated repression of the firefly luciferase gene can be measured by using the Renilla luciferase activity as a reference.

Figure 13: ShRNA-mediated inhibition of luciferase expression in mice.

A: Firefly luciferase activity in mice in the absence (delta shFluc, black bars) or presence of the U6- (U6-shFluc, white bars) and H1-shRNA transgenes (H1-shFluc, grey bars), respectively. All mice carried the firefly and the Renilla luciferase transgenes. Relative values of Firefly luciferase activitiy in different organs are given as indicated. All values of Fluc activity were normalized by using the Rluc activity for reference.

B: Efficiency of shRNA-mediated repression of firefly luciferase expression in mice. Percentages of U6- (white bars) and H1-shRNA mediated (grey bars) repression of Firefly luciferase activity are shown. ND: not determined.

Detailed Description of the Invention

The term "living organisms" according to the present invention relates to multi-cell organisms which can be living vertebrates (hereinafter shortly referred to as "vertrebrates") such as mammals (e.g. non-human animals such as rodents (including mice and rats, etc.; and humans) or non-mammals (e.g. fish) or can be invertebrates such as insects or worms, or can be plants (higher plants, algi or fungi). Most preferred living organisms are vertebrates, in particular mice and fish.

"Tissue culture" according to the present invention refers to parts of the above defined living organisms (including organs and the like) which are cultured *in vitro*.

"Cell culture" according to the present invention includes cells isolated from the above defined living organisms which are cultured *in vitro*. These cells can be transformed (immortalized) or untransformed (directly derived from living organisms; primary cell culture).

The expression vector according to the invention of the present application is suitable for stable integration into the living organism or into cells of the cell culture (vectors for transient integration are also contemplated). It is moreover preferred that it contains homologous sequences suitable for targeted integration at a defined locus, preferably at a polymerase II dependent locus of the living organisms or cells of the cell culture. Such polymerase II dependent loci include, but are not limited to, Rosa26 locus (the murine Rosa26 locus being depicted in SEQ ID NO:1), collagen, RNA polymerase, actin and HPRT.

The expression vector may further contain functional sequences selected from splice acceptor sequences (such as a splice acceptor of adenovirus, etc.), polyadenylation sites (such as synthetic polyadenylation sites, the

polyadenylation site of human growth hormones, or the like), selectable marker sequences (such as the neomycin phosphotransferase gene of *E. coli* transposon, etc.), etc.

The ubiquitous promoter in the vector according to the invention is preferably selected from polymerase I, II and III dependent promoters, preferably is a polymerase II or III dependent promoter including, but not limited to, a CMV promoter, a CAGGS promoter, a snRNA promoter such as U6, a RNAse P RNA promoter such as H1, a tRNA promoter, a 7SL RNA promoter, a 5 S rRNA promoter, etc.

The ubiquitous promoter can be a constitutive promoter, or can be an inducible promoter. Suitable inducible promoters are the above-mentioned polymerase I, II and III dependent promoters containing an operator sequence including, but not limited to, tet, Gal4, lac, etc.

The expression vector of the invention is suitable for the following particularly preferred approaches (for constitutive and inducible expression):

A. a Pol III dependent promoter (constitutive U6, H1 or the like) driven shRNA construct (to be integrated into a ubiquitously active Pol II dependent locus (see Fig. 2);
B. a Pol III dependent promoter (inducible U6, H1 or the like) driven shRNA construct (to be integrated into a ubiquitously active Pol II dependent locus (Fig 3 and 4)); or
C. a polymerase II (Pol II) dependent promoter (inducible CMV or the like) driven shRNA construct (to be integrated into a ubiquitously active Pol II dependent locus (Fig. 5 and 6)).

The ShRNA within the vector of the invention preferably comprises

(I) at least one DNA segment A-B-C wherein

A is a 15 to 35, preferably 19 to 29 bp DNA sequence being at least 95%, preferably 100% complementary to the gene to be knocked down (e.g. firefly luciferase, p53, etc.);

B is a spacer DNA sequence having 5 to 9 bp forming the loop of the expressed RNA hair pin molecule, and

C is a 15 to 35, preferably 19 to 29 bp DNA sequence being at least 85% complementary to the sequence A; and

(II) a stop and/or polyadenylation sequence.

Suitable shRNA sequences for the knock down of a given target gene are well known in the art (see e.g. the particular shRNA sequences mentioned in Tables 1 and 2 below) or can readily be determined by the skilled artesian.

Table 1:

target gene	shRNA sequence /SEQ ID NO	Reference
CDH-1 p53 CDC20	TgagaagtctcccagtcagTTCAAGAGActgactgggagacttctca (19) GactccagtggtaatctacTTCAAGAGAgtagattaccactggagtc (20) CggcaggactccgggccgaTTCAAGAGAtcggcccggagtcctgccg (21)	Brummelkamp et al., Science, 296: 550-3 (2002).
CYLD	CctcatgcagttctctttgTTCAAGAGAcaaagagaactgcatgagg (22)	Kovalenko et al, Nature, 424:801-5 (2003).
Ras-Gap	AagatgaagccactccctatttCAAGAGAaaatagggagtggcttcatctt (23)	Kunath et al., Nature Biotechnology, 21:559-561 (2003).
tubulin	GacagagccaagtggactcACAgagtccacttggctctgtc (24)	Yu et al., PNAS, 99: 6047-52 (2002)
lamin	Ctggacttccagaagaacattcgtgttcttctggaagtccag (25)	Paul et al., Nature Biotechnology, 20:505-8 (2002).

Table 2 shRNA sequences known from Brummelkamp et al., Nature, 424:797-801 (2003):

Target Gene	shRNA Sequence / SEQ ID NO
UBIQUITIN CARBOXYL-TERMINAL HYDROLASE 12	GAGATTGGTCCAGAACAGTTTCAAGAGAACTGTTCTGGACCAATCTC (26) GCCCTTCCGATCATGGTAGTTCAAGAGACTACCATGATCGGAAGGGC (27) TCTTTAGAATTCTTAAGTATTCAAGAGATACTTAAGAATTCTAAAGA (28) CATTAGCTATATCAACATGTTCAAGAGACATGTTGATATAGCTAATG (29)
UBIQUITIN CARBOXYL-TERMINAL HYDROLASE 11	ACCACAAACGGCGGAACGATTCAAGAGATCGTTCCGCCGTTTGTGGT (30) GAGGGTCTTGGAGGTCTTCTTCAAGAGAGAAGACCTCCAAGACCCTC (31) GTCCATGCCCAGCCGTACATTCAAGAGATGTACGGCTGGGCATGGAC (32) GCTGGACACCCTCGTGGAGTTCAAGAGACTCCACGAGGGTGTCCAGC (33)
UBIQUITIN CARBOXYL-TERMINAL HYDROLASE 10	GAATATCAGAGAATTGAGTTTCAAGAGAACTCAATTCTCTGATATTC (34) TGGACTTCATGAGGAAATGTTCAAGAGACATTTCCTCATGAAGTCCA (35) TATTGAATATCCTGTGGACTTCAAGAGAGTCCACAGGATATTCAATA (36) TTGTACTGAGAGAAACTGCTTCAAGAGAGCAGTTTCTCTCAGTACAA (37)
HAUSP	GATCAATGATAGGTTTGAATTCAAGAGATTCAAACCTATCATTGATC (38) GGAGTTTGAGAAGTTTAAATTCAAGAGATTTAAACTTCTCAAACTCC (39) GAACTCCTCGCTTGCTGAGTTCAAGAGACTCAGCAAGCGAGGAGTTC (40) CCGAATTTAACAGAGAGAATTCAAGAGATTCTCTCTGTTAAATTCGG (41)
UBIQUITIN CARBOXYL-TERMINAL HYDROLASE 8	GACAGCAGAAGAATGCAGATTCAAGAGATCTGCATTCTTCTGCTGTC (42) ATAAAGCTCAACGAGAACCTTCAAGAGAGGTTCTCGTTGAGCTTTAT (43) GGTGAAGTGGCAGAAGAATTTCAAGAGAATTCTTCTGCCACTTCACC (44) GTATTGCAGTAATCATCACTTCAAGAGAGTGATGATTACTGCAATAC (45)
FLJ10785	GATATGGGGTTCCATGTCATTCAAGAGATGACATGGAACCCCATATC (46) GGAGACATGGTTCTTAGTGTTCAAGAGACACTAAGAACCATGTCTCC (47) AGCACCAAGTTCGTCTCAGTTCAAGAGACTGAGACGAACTTGGTGCT (48) GATGCAACACTGAAAGAACTTCAAGAGAGTTCTTTCAGTGTTGCATC (49)
KIAA0710	GTCAATGGCAGTGATGATATTCAAGAGATATCATCACTGCCATTGAC (50) CCTGCTAGCTGCCTGTGGCTTCAAGAGAGCCACAGGCAGCTAGCAGG (51) CCACCTTTGCCAGAAGGAGTTCAAGAGACTCCTTCTGGCAAAGGTGG (52) CCCTATTGAGGCAAGTGTCTTCAAGAGAGACACTTGCCTCAATAGGG (53)
FLJ12552/ FLJ14256	GAAGGAAAACTTGCTGACGTTCAAGAGACGTCAGCAAGTTTTCCTTC (54) CTCACCTGGGTCCATGAGATTCAAGAGATCTCATGGACCCAGGTGAG (55) GCTGTCTTACCGTGTGGTCTTCAAGAGAGACCACACGGTAAGACAGC (56) CCTGGACCGCATGTATGACTTCAAGAGAGTCATACATGCGGTCCAGG (57)
KIAA1203	GTCAATGGCAGTGATGATATTCAAGAGATATCATCACTGCCATTGAC (58) CCTGCTAGCTGCCTGTGGCTTCAAGAGAGCCACAGGCAGCTAGCAGG (59) CCACCTTTGCCAGAAGGAGTTCAAGAGACTCCTTCTGGCAAAGGTGG (60) CCCTATTGAGGCAAGTGTCTTCAAGAGAGACACTTGCCTCAATAGGG (61)

FLJ23277	GGAAATCCGAATTGCTTGGTTCAAGAGACCAAGCAATTCGGATTTCC	(62)
	CACATTTCTTCAAGTGTGGTTCAAGAGACCACACTTGAAGAAATGTG	(63)
	CAGCAGGATGCTCAAGAATTTCAAGAGAATTCTTGAGCATCCTGCTG	(64)
	GCTGAATACCTACATTGGCTTCAAGAGAGCCAATGTAGGTATTCAGC	(65)
FLJ14914 (similar to UBP4)	GGGCTTGTGCCTGGCCTTGTTCAAGAGACAAGGCCAGGCACAAGCCC	(66)
	GCCTTGTCCTGCCAAGAAGTTCAAGAGACTTCTTGGCAGGACAAGGC	(67)
	GATTGAAGCCAAGGGAACGTTCAAGAGACGTTCCCTTGGCTTCAATC	(68)
	TGGCGCCTGCTCCCCATCTTTCAAGAGAAGATGGGGAGCAGGCGCCA	(69)
UBIQUITIN CARBOXYL-TERMINAL HYDROLASE ISOZYME L5	GAACCAGCAGGCTCTGTGGTTCAAGAGACCACAGAGCCTGCTGGTTC	(70)
	GGAAGCATAATTATCTGCCTTCAAGAGAGGCAGATAATTATGCTTCC	(71)
	AGAAGAAGATGCTTTTCACTTCAAGAGAGTGAAAAGCATCTTCTTCT	(72)
	CTTGCAGAGGAGGAACCCATTCAAGAGATGGGTTCCTCCTCTGCAAG	(73)
UBIQUITIN CARBOXYL-TERMINAL HYDROLASE ISOZYME L3	GCAAACAATCAGCAATGCCTTCAAGAGAGGCATTGCTGATTGTTTGC	(74)
	TTGGACTGATTCATGCTATTTCAAGAGAATAGCATGAATCAGTCCAA	(75)
	CTGGCAATTCGTTGATGTATTCAAGAGATACATCAACGAATTGCCAG	(76)
	TTAGATGGGCGGAAGCCATTTCAAGAGAATGGCTTCCGCCCATCTAA	(77)
UBIQUITIN CARBOXYL-TERMINAL HYDROLASE ISOZYME L1	GAGGAGTCTCTGGGCTCGGTTCAAGAGACCGAGCCCAGAGACTCCTC	(78)
	GAGCTGAAGGGACAAGAAGTTCAAGAGACTTCTTGTCCCTTCAGCTC	(79)
	TGTCGGGTAGATGACAAGGTTCAAGAGACCTTGTCATCTACCCGACA	(80)
	CACAGCTGTTCTTCTGTTCTTCAAGAGAGAACAGAAGAACAGCTGTG	(81)
KIAA1891 / FLJ25263	GTGGAAGCCTTTACAGATCTTCAAGAGAGATCTGTAAAGGCTTCCAC	(82)
	CAACAGCTGCCTTCATCTGTTCAAGAGACAGATGAAGGCAGCTGTTG	(83)
	CCATAGGCAGTCCTCCTAATTCAAGAGATTAGGAGGACTGCCTATGG	(84)
	TGTATCACTGCCACTGGTTTTCAAGAGAAACCAGTGGCAGTGATACA	(85)
FLJ14528 (similar to UBP8)	CATGTTGGGCAGCTGCAGCTTCAAGAGAGCTGCAGCTGCCCAACATG	(86)
	CACAACTGGAGACCTGAAGTTCAAGAGACTTCAGGTCTCCAGTTGTG	(87)
	GTATGCCTCCAAGAAAGAGTTCAAGAGACTCTTTCTTGGAGGCATAC	(88)
	CTTCACAGTACATTTCTCTTTCAAGAGAAGAGAAATGTACTGTGAAG	(89)
U4/U6 TRI SNRNP 65 kDa protein	GTACTTTCAAGGCCGGGGTTTCAAGAGAACCCCGGCCTTGAAAGTAC	(90)
	CTTGGACAAGCAAGCCAAATTCAAGAGATTTGGCTTGCTTGTCCAAG	(91)
	GACTATTGTGACTGATGTTTTCAAGAGAAACATCAGTCACAATAGTC	(92)
	GGAGAACTTTCTGAAGCGCTTCAAGAGAGCGCTTCAGAAAGTTCTCC	(93)
XM_089437	GACGAGAGAAACCTTCACCTTCAAGAGAGGTGAAGGTTTCTCTCGTC	(94)
	ACATTATTCTACATTCTTTTTCAAGAGAAAAGAATGTAGAATAATGT	(95)
	AGATTCGCAAATGGATGTATTCAAGAGATACATCCATTTGCGAATCT	(96)
	CATTCCCACCATGAGTCTGTTCAAGAGACAGACTCATGGTGGGAATG	(97)
KIAA1453	GATCGCCCGACACTTCCGCTTCAAGAGAGCGGAAGTGTCGGGCGATC	(98)
	CCAGCAGGCCTACGTGCTGTTCAAGAGACAGCACGTAGGCCTGCTGG	(99)
	GCCAGCTCCTCCACAGCACTTCAAGAGAGTGCTGTGGAGGAGCTGGC	(100)
	CGCCGCCAAGTGGAGCAGATTCAAGAGATCTGCTCCACTTGGCGGCG	(101)

FLJ12697	GAAGATGCCCATGAATTCCTTCAAGAGAGGAATTCATGGGCATCTTC (102)
	CAAACAGGCTGCGCCAGGCTTCAAGAGAGCCTGGCGCAGCCTGTTTG (103)
	ACGGCCTAGCGCCTGATGGTTCAAGAGACCATCAGGCGCTAGGCCGT (104)
	CTGTAACCTCTCTGATCGGTTCAAGAGACCGATCAGAGAGGTTACAG (105)
UBIQUITIN SPECIFIC PROTEASE 18 (USP18)	TCTGTCAGTCCATCCTGGCTTCAAGAGAGCCAGGATGGACTGACAGA (106)
	TGAAGCGAGAGTCTTGTGATTCAAGAGATCACAAGACTCTCGCTTCA (107)
	GATGGAGTGCTAATGGAAATTCAAGAGATTTCCATTAGCACTCCATC (108)
	CCTTCAGAGATTGACACGCTTCAAGAGAGCGTGTCAATCTCTGAAGG (109)
UBIQUITIN CARBOXYL-TERMINAL HYDROLASE 20	CCTGACCACGTTCCGACTGTTCAAGAGACAGTCGGAACGTGGTCAGG (110)
	GAGTTCCTTCGCTGCCTGATTCAAGAGATCAGGCAGCGAAGGAACTC (111)
	GACTGCCTTGCTGCCTTCTTTCAAGAGAAGAAGGCAGCAAGGCAGTC (112)
	CGCCGAGGGCTACGTACTCTTCAAGAGAGAGTACGTAGCCCTCGGCG (113)
UBIQUITIN CARBOXYL-TERMINAL HYDROLASE 24	GGCGAGAAGAAAGGACTGTTTCAAGAGAACAGTCCTTTCTTCTCGCC (114)
	GGACGAGAATTGATAAAGATTCAAGAGATCTTTATCAATTCTCGTCC (115)
	GCACGAGAATTTGGGAATCTTCAAGAGAGATTCCCAAATTCTCGTGC (116)
	CTACTTCATGAAATATTGGTTCAAGAGACCAATATTTCATGAAGTAG (117)
KIAA1594	GATAACAGCTTCTTGTCTATTCAAGAGATAGACAAGAAGCTGTTATC (118)
	GAGAATAGGACATCAGGGCTTCAAGAGAGCCCTGATGTCCTATTCTC (119)
	CTTGGAAGACTGAACCTGTTTCAAGAGAACAGGTTCAGTCTTCCAAG (120)
	CAACTCCTTTGTGGATGCATTCAAGAGATGCATCCACAAAGGAGTTG (121)
KIAA1350	GATGTTGTCTCCAAATGCATTCAAGAGATGCATTTGGAGACAACATC (122)
	CGTGGGGACTGTACCTCCCTTCAAGAGAGGGAGGTACAGTCCCCACG (123)
	GTACAGCTTCAGAACCAAGTTCAAGAGACTTGGTTCTGAAGCTGTAC (124)
UBIQUITIN CARBOXYL-TERMINAL HYDROLASE 25	GATGATCTTCAGAGAGCAATTCAAGAGATTGCTCTCTGAAGATCATC (125)
	GGAACATCGGAATTTGCCTTTCAAGAGAAGGCAAATTCCGATGTTCC (126)
	GAGCTAGTGAGGGACTCTTTTCAAGAGAAAGAGTCCCTCACTAGCTC (127)
	GCAGGGTTCTTTAAGGCAATTCAAGAGATTGCCTTAAAGAACCCTGC (128)
UBIQUITIN CARBOXYL-TERMINAL HYDROLASE 16	TCGATGATTCCTCTGAAACTTCAAGAGAGTTTCAGAGGAATCATCGA (129)
	GATAATGGAAATATTGAACTTCAAGAGAGTTCAATATTTCCATTATC (130)
	GTTCTTCATTTAAATGATATTCAAGAGATATCATTTAAATGAAGAAC (131)
	GTTAACAAACACATAAAGTTTCAAGAGAACTTTATGTGTTTGTTAAC (132)
USP9X	GTTAGAGAAGATTCTTCGTTTCAAGAGAACGAAGAATCTTCTCTAAC (133)
	GTTGATTGGACAATTAAACTTCAAGAGAGTTTAATTGTCCAATCAAC (134)
	GGTTGATACCGTAAAGCGCTTCAAGAGAGCGCTTTACGGTATCAACC (135)
	GCAATGAAACGTCCAATGGTTCAAGAGACCATTGGACGTTTCATTGC (136)
USP9Y	AGCTAGAGAAAATTCTTCGTTCAAGAGACGAAGAATTTTCTCTAGCT (137)
	GATCCTATATGATGGATGATTCAAGAGATCATCCATCATATAGGATC (138)
	GTTCTTCTTGTCAGTGAAATTCAAGAGATTTCACTGACAAGAAGAAC (139)
	CTTGAGCTTGAGTGACCACTTCAAGAGAGTGGTCACTCAAGCTCAAG (140)

UBIQUITIN CARBOXYL-TERMINAL HYDROLASE 5	GACCGGCCAGCGAGTCTACTTCAAGAGAGTAGACTCGCTGGCCGGTC (141) GGACCTGGGCTACATCTACTTCAAGAGAGTAGATGTAGCCCAGGTCC (142) CTCTGTGGTCCAGGTGCTCTTCAAGAGAGAGCACCTGGACCACAGAG (143) GACCACACGATTTGCCTCATTCAAGAGATGAGGCAAATCGTGTGGTC (144)
UBIQUITIN CARBOXYL-TERMINAL HYDROLASE 26	TGGCTTGTTTATTGAAGGATTCAAGAGATCCTTCAATAAACAAGCCA (145) GTGAATTTGGGGAAGATAATTCAAGAGATTATCTTCCCCAAATTCAC (146) CGCTATAGCTTGAATGAGTTTCAAGAGAACTCATTCAAGCTATAGCG (147) GATATCCTGGCTCCACACATTCAAGAGATGTGTGGAGCCAGGATATC (148)
KIAA1097	GAGCCAGTCGGATGTAGATTTCAAGAGAATCTACATCCGACTGGCTC (149) GTAAATTCTGAAGGCGAATTTCAAGAGAATTCGCCTTCAGAATTTAC (150) GCCCTCCTAAATCAGGCAATTCAAGAGATTGCCTGATTTAGGAGGGC (151) GTTGAGAAATGGAGTGAAGTTCAAGAGACTTCACTCCATTTCTCAAC (152)
UBIQUITIN SPECIFIC PROTEASE 22 (USP22)	GCTTGGAAAATGCAAGGCGTTCAAGAGACGCCTTGCATTTTCCAAGC (153) CTGCATCATAGACCAGATCTTCAAGAGAGATCTGGTCTATGATGCAG (154) GATCACCACGTATGTGTCCTTCAAGAGAGGACACATACGTGGTGATC (155) TGACAACAAGTATTCCCTGTTCAAGAGACAGGGAATACTTGTTGTCA (156)
UBIQUITIN-SPECIFIC PROCESSING PROTEASE 29	GAAATATAAGACAGATTCCTTCAAGAGAGGAATCTGTCTTATATTTC (157) CCCATCAAGTTTAGAGGATTTCAAGAGAATCCTCTAAACTTGATGGG (158) GGTGTCCCATGGGAATATATTCAAGAGATATATTCCCATGGGACACC (159) GAATGCCGACCTACAAAGATTCAAGAGATCTTTGTAGGTCGGCATTC (160)
CYLD	CAGTTATATTCTGTGATGTTTCAAGAGAACATCACAGAATATAACTG (161) GAGGTGTTGGGGACAAAGGTTCAAGAGACCTTTGTCCCCAACACCTC (162) GTGGGCTCATTGGCTGAAGTTCAAGAGACTTCAGCCAATGAGCCCAC (163) GAGCTACTGAGGACAGAAATTCAAGAGATTTCTGTCCTCAGTAGCTC (164)
UBIQUITIN CARBOXYL-TERMINAL HYDROLASE 2	TCAGCAGGATGCTCAGGAGTTCAAGAGACTCCTGAGCATCCTGCTGA (165) GAAGTTCTCCATCCAGAGGTTCAAGAGACCTCTGGATGGAGAACTTC (166) GCCGGTCCCCACCAGCAGCTTCAAGAGAGCTGCTGGTGGGGACCGGC (167) CACTCGGGAGTTGAGAGATTTCAAGAGAATCTCTCAACTCCCGAGTG (168)
UBIQUITIN SPECIFIC PROTEASE 3 (USP3)	GCCCTTGGGTCTGTTTGACTTCAAGAGAGTCAAACAGACCCAAGGGC (169) CTCAACACTAAACAGCAAGTTCAAGAGACTTGCTGTTTAGTGTTGAG (170) GATTTCATTGGACAGCATATTCAAGAGATATGCTGTCCAATGAAATC (171) CATGGGGCACCAACTAATTTTCAAGAGAAATTAGTTGGTGCCCCATG (172)
UBIQUITIN CARBOXYL-TERMINAL HYDROLASE 23	GGTGTCTCTGCGGGATTGTTTCAAGAGAACAATCCCGCAGAGACACC (173) AGTTCAGTAGGTGTAGACTTTCAAGAGAAGTCTACACCTACTGAACT (174) GAGTTCCTGAAGCTCCTCATTCAAGAGATGAGGAGCTTCAGGAACTC (175) GGATTTGCTGGGGGCAAGGTTCAAGAGACCTTGCCCCCAGCAAATCC (176)
UBP-32.7	CTCAGAAAGCCAACATTCATTCAAGAGATGAATGTTGGCTTTCTGAG (177) CGCATTGTAATAAGAAGGTTTCAAGAGAACCTTCTTATTACAATGCG (178) GGGAGGAAAATGCAGAAATTTCAAGAGAATTTCTGCATTTTCCTCCC (179) TTACAAATTTAGGAAATACTTCAAGAGAGTATTTCCTAAATTTGTAA (180)

HOMO SAPIENS	GTTATGAATTGATATGCAGTTCAAGAGACTGCATATCAATTCATAAC (181)
UBIQUITIN SPE-	GTGATAACACAACTAATGGTTCAAGAGACCATTAGTTGTGTTATCAC (182)
CIFIC PROTEASE	GTAGAGGAGAGTTCTGAAATTCAAGAGATTTCAGAACTCTCCTCTAC (183)
13 (ISOPEP-	GCCTCTAATCCTGATAAGGTTCAAGAGACCTTATCAGGATTAGAGGC (184)
TIDASE T-3)	
UBIQUITIN	GATGATCTTCAGGCTGCCATTCAAGAGATGGCAGCCTGAAGATCATC (185)
CARBOXYL-	GTATGGACAAGAGCGTTGGTTCAAGAGACCAACGCTCTTGTCCATAC (186)
TERMINAL	CGAACCCTTCTGGAACAGTTTCAAGAGAACTGTTCCAGAAGGGTTCG (187)
HYDROLASE 28	GTGGCATGAAGATTATAGTTTCAAGAGAACTATAATCTTCATGCCAC (188)
UBIQUITIN	GGTGAACAAGGACAGTATCTTCAAGAGAGATACTGTCCTTGTTCACC (189)
CARBOXYL-	GCAATAGAGGATGATTCTGTTCAAGAGACAGAATCATCCTCTATTGC (190)
TERMINAL	TCTGTGAATGCCAAAGTTCTTCAAGAGAGAACTTTGGCATTCACAGA (191)
HYDROLASE 14	CACACCAGGGAAGGTCTAGTTCAAGAGACTAGACCTTCCCTGGTGTG (192)
DUB1	GCAGGAAGATGCCCATGAATTCAAGAGATTCATGGGCATCTTCCTGC (193)
	GAATGTGCAATATCCTGAGTTCAAGAGACTCAGGATATTGCACATTC (194)
	TGGATGATGCCAAGGTCACTTCAAGAGAGTGACCTTGGCATCATCCA (195)
	GCTCCGTGCTAAACCTCTCTTCAAGAGAGAGAGGTTTAGCACGGAGC (196)
MOUSE USP27	GCCTCCACCTCAACAGAGGTTCAAGAGACCTCTGTTGAGGTGGAGGC (197)
HOMOLOG	CTGCATCATAGACCAAATCTTCAAGAGAGATTTGGTCTATGATGCAG (198)
	GATCACTACATACATTTCCTTCAAGAGAGGAAATGTATGTAGTGATC (199)
	GTAAAGAGAGCAGAATGAATTCAAGAGATTCATTCTGCTCTCTTTAC (200)
UBIQUITIN	CGCGGGGCGCAGTGGTATCTTCAAGAGAGATACCACTGCGCCCCGCG (201)
CARBOXYL-	CAGAAGGCAGTGGGGAAGATTCAAGAGATCTTCCCCACTGCCTTCTG (202)
TERMINAL	GCCTGGGAGAATCACAGGTTTCAAGAGAACCTGTGATTCTCCCAGGC (203)
HYDROLASE 4	ACCAGACAAGGAAATACCCTTCAAGAGAGGGTATTTCCTTGTCTGGT (204)
TRE-2	CACATCCACCACATCGACCTTCAAGAGAGGTCGATGTGGTGGATGTG (205)
	GTCACAACCCAAGACCATGTTCAAGAGACATGGTCTTGGGTTGTGAC (206)
	CTCAACAGGACAAATCCCATTCAAGAGATGGGATTTGTCCTGTTGAG (207)
	TAGATCAATTATTGTGGATTTCAAGAGAATCCACAATAATTGATCTA (208)
UBIQUITIN CAR-	GGAACACCTTATTGATGAATTCAAGAGATTCATCAATAAGGTGTTCC (209)
BOXYL-TERMINAL	CTTTAACAGAAATTGTCTCTTCAAGAGAGAGACAATTTCTGTTAAAG (210)
HYDROLASE 15	CCTATGCAGTACAAAGTGGTTCAAGAGACCACTTTGTACTGCATAGG (211)
(UNPH-2).	GATCTTTTCTTGCTTTGGATTCAAGAGATCCAAAGCAAGAAAAGATC (212)
KIAA1372	CAGCATCCTTCAGGCCTTATTCAAGAGATAAGGCCTGAAGGATGCTG (213)
	GATAGTGACTCGGATCTGCTTCAAGAGAGCAGATCCGAGTCACTATC (214)
	GACATCACAGCCCGGGAGTTTCAAGAGAACTCCCGGGCTGTGATGTC (215)
	GGACACAGCCTATGTGCTGTTCAAGAGACAGCACATAGGCTGTGTCC (216)
BRCA1	GTGGAGGAGATCTACGACCTTCAAGAGAGGTCGTAGATCTCCTCCAC (217)
ASSOCIATED	CTCTTGTGCAACTCATGCCTTCAAGAGAGGCATGAGTTGCACAAGAG (218)
PROTEIN-1	ACAGGGCCCCTGCAGCCTCTTCAAGAGAGAGGCTGCAGGGGCCCTGT (219)
	GAAGACCTGGCGGCAGGTGTTCAAGAGACACCTGCCGCCAGGTCTTC (220)

A preferred embodiment of the method (2) of the invention concerns the following steps:

1. Generation of the short hairpin DNA containing the antisense- and sense-strand of the coding region of a gene (e.g. firefly luciferase; p53). Antisense and sense-strand are separated by a spacer of 5 to 9 bp (?).
2. Generation of constructs for the expression of the above mentioned shRNA under the control of a constitutive or inducible promoter (Pol II or Pol III dependent).
3. Insertion of the mentioned expression constructs into a ubiquitous expressed Pol II dependent locus by homologous recombination in ES cells.
4. Analysis of the constitutive and inducible inhibition of gene expression (e.g. firefly luciferase; p53) in ES cells (e.g. through Western blot analysis).
5. Generation of mice using the mentioned ES cells and analysis of the inhibition of gene expression in several tissues (e.g. firefly luciferase; p53; e.g. through Western blot analysis).

The vector according to embodiment (4) of the invention is suitable for stable or transient integration. Said vector is suitable for gene transfer.

The technology of the present application provides for the following advantages:

(i) A stable and body wide inhibition of gene expression by generating transgenic animals (such as mice).

(ii) A reversible inhibition of gene expression using the inducible constructs.

The invention is furthermore described by the following examples which are, however, not to be construed so as to limit the invention.

Examples

Methods

Plasmid construction: All plasmid constructs were generated by standard DNA cloning methods. The U6- and the H1-promoter fragments were amplified from human genomic DNA. The Renilla luciferase (Rluc) and firefly luciferase (Fluc) coding regions (Promega) were placed 3' of the adenovirus splice acceptor site (Friedrich and Soriano, Genes Dev. 9:1513-23 (1991); see SEQ ID NOs: 3, 5 and 7) to facilitate transcription from the endogenous *rosa26* promoter. For homologous recombination in embryonic stem cells, the expression cassettes were flanked by homology regions for the *Rosa26 locus* (see SEQ ID NO: 2).

Cell culture: Culture and targeted mutagenesis of F1 (C57BL/6 x 129Sv/Ev) ES cells were carried out as previously described (Hogan,B., Beddington,R., Costantini,F. and Lacy,E. (1994). *A Laboratory Manual. In Manipulating the Mouse Embryo.* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY.), pp. 253-289.). For Cre-mediated deletion, ES cells were transiently transfected with 5 µg of the cre expression plasmid pCAGGScre (SEQ ID NO: 18). 24 h after transfection, 1000 ES cells were plated on a 10 cm dish. Individual clones were isolated and analyzed for cre-mediated recombination by Southern blot using standard protocols.

For induction, cells were cultivated in cell culture medium containing 1 µg/ml Doxycycline (Sigma) for 2 days.

For transient transfection of cells, mixtures of 350 ng supercoiled plasmid DNA pre-complexed with the FuGene6 transfection reagent (Roche Diagnostics GmbH, Mannheim, Germany) were added to the cells in 250 µl medium, according to the manufacturers protocol. Each plasmid mixture contained 50 ng of the beta-galactosidase expression vector CMV-lacZ (SEQ ID NO: 17), 100 ng of CAGGS-Fluc (SEQ ID NO: 15), 100 ng of the tet inducible H1shRNA vector (SEQ ID NO: 11, 12 or 13), and pUC19 to a total amount of 350 ng DNA. For each sample to be tested four individual wells were transfected. One

day after the addition of the DNA preparations, the cell culture medium was exchanged.

Luciferase measurement: Stably transfected cells: 25.000 cells were plated on a 24 well plate and analyzed 48h later. Luciferase activity was detected using the Dual-Glo™ Luciferase Assay System (Promega) and a Lumat LB 9507 (EG&G Berthold) according to the manufacturers protocols.
Transiently transfected cells: The cells were lysed 48 hours after transfection with 100 µl/well of lysate reagent supplemented with protease inhibitors (ß-galactosidase reporter gene assay kit, Roche Diagnostics). The lysates were centrifuged and 20 µl were used to determine the µ-galactosidase activities using the ß-galactosidase reporter gene assay (Roche Diagnostics) according to the manufacturers protocol in a Lumat LB 9507 luminometer (Berthold). To measure Luciferase activity, 20 µl of lysate was diluted into 250 µl assay buffer (50mM glycylglycin, 5mM $MgCl_2$, 5mM ATP) and the relative light units (RLU) were counted in a Lumat LB 9507 luminometer after addition of 100 µl of a 1 mM Luciferin (Roche Diagnostics) solution.

Generation of chimeric mice: Recombinant ES cells were injected into blastocysts from Balb/C mice and chimeric mice were obtained upon transfer of blastocysts into pseudopregnant females using standard protocols (Hogan et al. Manipulating the Mouse Embryo: A Laboratory Manual. *Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY.* 253-289 (1994)).

Luciferase measurement in organs: Organs were homogenized at 4 °C in lysis buffer (0.1M KH_2PO_4, 1mM DTT, 0.1% Triton® X-100) using a tissue grinder. Spin for 5' at 2000xg (4 °C) to pellet debris and assay supernatant for Luc activities using the Dual Luciferase Assay (Promega, Inc.) according to the manufacturer protocol.

Example 1

The firefly luciferase gene along with a splice acceptor sequence (Friedrich and Soriano, Genes Dev. 9:1513-23 (1991)) was inserted into the first allele of the *rosa26 locus* by homologous recombination in ES cells. The shRNA expression cassettes under the control of the H1 or the U6 promoter and a Renilla luciferase gene were inserted into the second allele of the rosa26 locus by homologous recombination. The Renilla luciferase gene was used as a reference to normalize the values of firefly luciferase activity.

Deleting the loxP-flanked shRNA expression cassette by transient transfection of a cre expression plasmid generated the negative control. Figure 7 shows the expression of the firefly luciferase in the absence and the presence of the shRNA expression cassette. Expression of the shRNA under the control of the H1 promoter resulted in a ~75% reduction of firefly luciferase activity, whereas the repression mediated by the U6-shRNA construct was ~60%.

Example 2

The shRNA expression cassette under the control of the U6 promoter containing tet-operator sequences, and a Renilla luciferase gene were inserted into the first allele of the rosa26 locus (figure 8 and 12; SEQ ID NOs:9 and 10). The firefly luciferase gene under the control of the endogenous *rosa26* promoter and a tet repressor expression cassette (Gossen and Bujard, PNAS. 89: 5547-5551 (1992)) was inserted into the second allele of *rosa26* by homologous recombination in ES cells (figure 8; SEQ ID NO:14). To determine the extent of shRNA-mediated gene silencing, the cells were treated for 2 days in the presence or in the absence of 1 µg/ml Doxycycline in the cell culture medium. Figure 10 shows the firefly luciferase activity measured in the cell extracts. Doxycycline inducible expression of the shRNA under the control of the $U6_{tetO1}$ promoter (SEQ ID NO:9) resulted in a ~30% reduction of firefly luciferase activity, whereas repression mediated by the $U6_{tetO2}$ -construct (SEQ ID NO 10) reached ~50%.

Example 3

NIH3T3 cells were transiently transfected with constructs expressing the luciferase and tet repressor genes (SEQ ID NOs:15 and 16) together with the shRNA constructs under the control of the H1 promoter containing tet-operator sequences (sequence ID NOs:11, 12 and 13; figure 9). Figure 11 shows the expression of firefly luciferase in the absence and in the presence of 1 µg/ml doxycycline. The highest degree of doxycycline inducible shRNA expression was achieved with the $H1_{tetO2/3'}$-promoter, resulting in a ~80% reduction of firefly luciferase activity.

Example 4

To examine the activity of the Rosa26/U6- and H1-shRNA transgenes in vivo, recombinant ES cells were injected into diploid blastocysts and chimeric mice were obtained upon transfer of blastocysts into pseudopregnant females. Figure 13 shows the activity of firefly luciferase in different organs in the presence and absence of the shRNA expression cassettes. The Renilla luciferase gene at the second Rosa26 allele served as a reference to normalize the values of firefly luciferase activity. Expression of the shRNA under the control of both, the U6 as well as the H1 promoter resulted in efficient repression of firefly luciferase activity in most organs (Fig. 13A), with the highest degree of reduction (>90%) in liver, heart, brain and muscle (Fig. 13B).

CLAIMS:

1. Use of an expression vector comprising a short hairpin RNA construct under control of a ubiquitous promoter for preparing an agent (shRNA) for constitutive and/or inducible gene knock down in a vertebrate, or in tissue culture or cells of a cell culture derived from a vertebrate.

2. The use of claim 1, wherein the expression vector
(i) is suitable for stabile integration into the genome of a vertebrate, or into the genome of the tissue culture or of cells of the cell culture; and/or
(ii) contains homologous sequences suitable for integration at a defined genomic locus through homologous recombination, preferably at a polymerase II dependent locus in the genome of the vertebrate, in the genome of the tissue culture or in the genome of the cells of the cell culture including embryonic cells; and/or
(iii) further contains functional sequences selected from splice acceptor sequences, polyadenylation sites, selectable marker sequences, etc.

3. The use of claim 2, wherein the polymerase II dependent locus is selected from a Rosa26, collagen, RNA polymerase, actin and HPRT locus.

4. The use according to any one of claims 1 to 3, wherein
(i) the ubiquitous promoter is selected from polymerase I, II and III dependent promoters, preferably is a polymerase II or III dependent promoter, most preferably is a CMV promoter, a CAGGS promoter, a snRNA promoter such as U6, a RNAse P RNA promoter such as H1, a tRNA promoter, a 7SL RNA promoter, a 5 S rRNA promoter, etc.; and/or
(ii) the ubiquitous promoter is a constitutive promoter, or is an inducible promoter, preferably a promoter containing an operator sequence selected from tet, Gal4, lac, etc.; and/or
(iii) said vertebrate is a non-human vertebrate, preferably is a mouse or fish.

5. The use according to any one of claims 1 to 4, wherein the expression vector is

(a) a Pol III dependent promoter, preferably constitutive H1 or U6, driven shRNA construct suitable to be integrated into a ubiquitously active Pol II dependent locus;

(b) a Pol III dependent promoter, preferably inducible U6 or H1, driven shRNA construct suitable to be integrated into a ubiquitously active Pol II dependent locus; or

(c) a Pol II dependent promoter, preferably inducible CMV, driven shRNA construct suitable to be integrated into a ubiquitously active Pol II dependent locus.

6. The use according to any one of claims 1 to 5, wherein the shRNA comprises

(I) at least one DNA segment A-B-C wherein

A is a 15 to 35, preferably 19 to 29 bp DNA sequence with at least 95%, preferably 100% complementarily to the gene to be knocked down;

B is a spacer DNA sequence having 5 to 9 bp forming the loop of the expressed RNA hair pin molecule, and

C is a 15 to 35, preferably 19 to 29 bp DNA sequence with at least 85% complementarily to the sequence A; and

(II) a stop and or polyadenylation sequence.

7. A method for constitutive and/or inducible gene knock down in a vertebrate, or in a tissue culture or cells of a cell culture derived from a vertebrate, which comprises stably integrating an expression vector as defined in any one of claims 1 to 6 into the genome of the vertebrate, of the tissue culture or of the cells of the cell culture.

8. The method of claim 7, wherein said vertebrate is a non-human vertebrate, preferably is a mouse or fish.

9. The method of claim 7 or 8, wherein the expression vector is integrated at a polymerase dependent locus of the living organism, tissue culture or cell culture.

10. The method according to any one of claim 7 to 9, wherein the method for constitutive and/or inducible gene knock down in a vertebrate comprises integrating the expression vector into ES cells of the vertebrate.

11. A vertebrate, or tissue or cell culture derived from a vertebrate having stably integrated, preferably at a polymerase II dependent locus of the vertebrate, tissue culture or cells of the cell culture, an expression vector as defined in any one of claims 1 to 6.

12. The vertebrate tissue or cell culture of claim 11, which is or is derived from a non-human vertebrate, preferably mouse or xfish.

13. An expression vector as defined in any one of claims 1 to 6.

- 1 / 8-

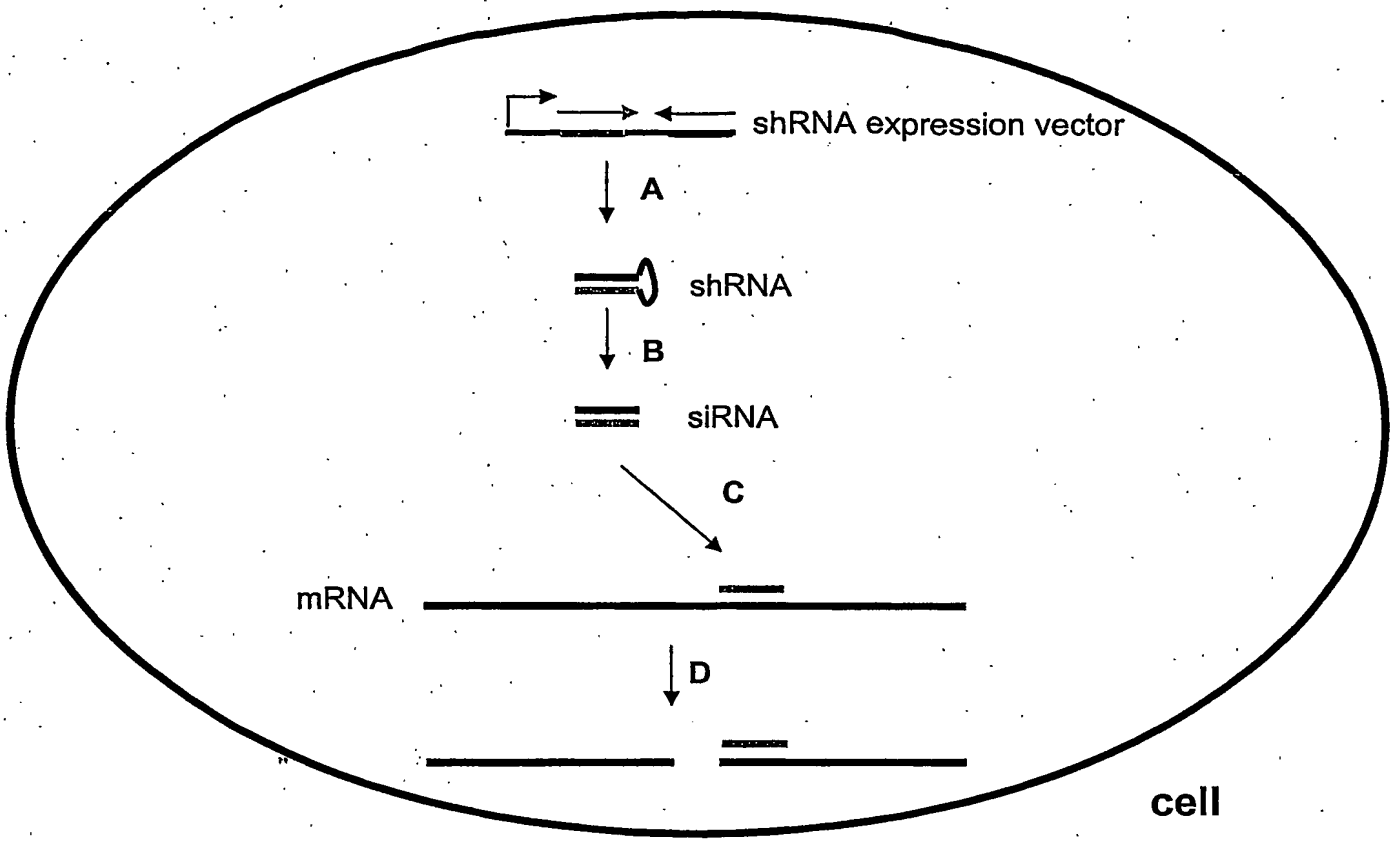
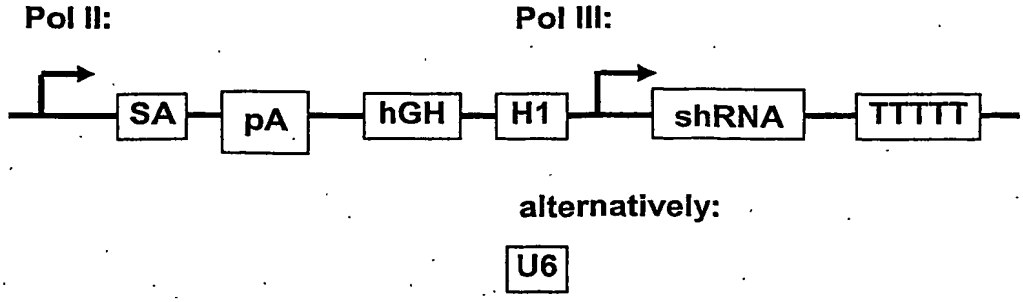

shRNA expression vector
A
shRNA
B
siRNA
C
mRNA
D
cell

Fig. 1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Pol III:
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pA
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H1
shRNA
TTTTT
alternatively:
U6

Fig. 2

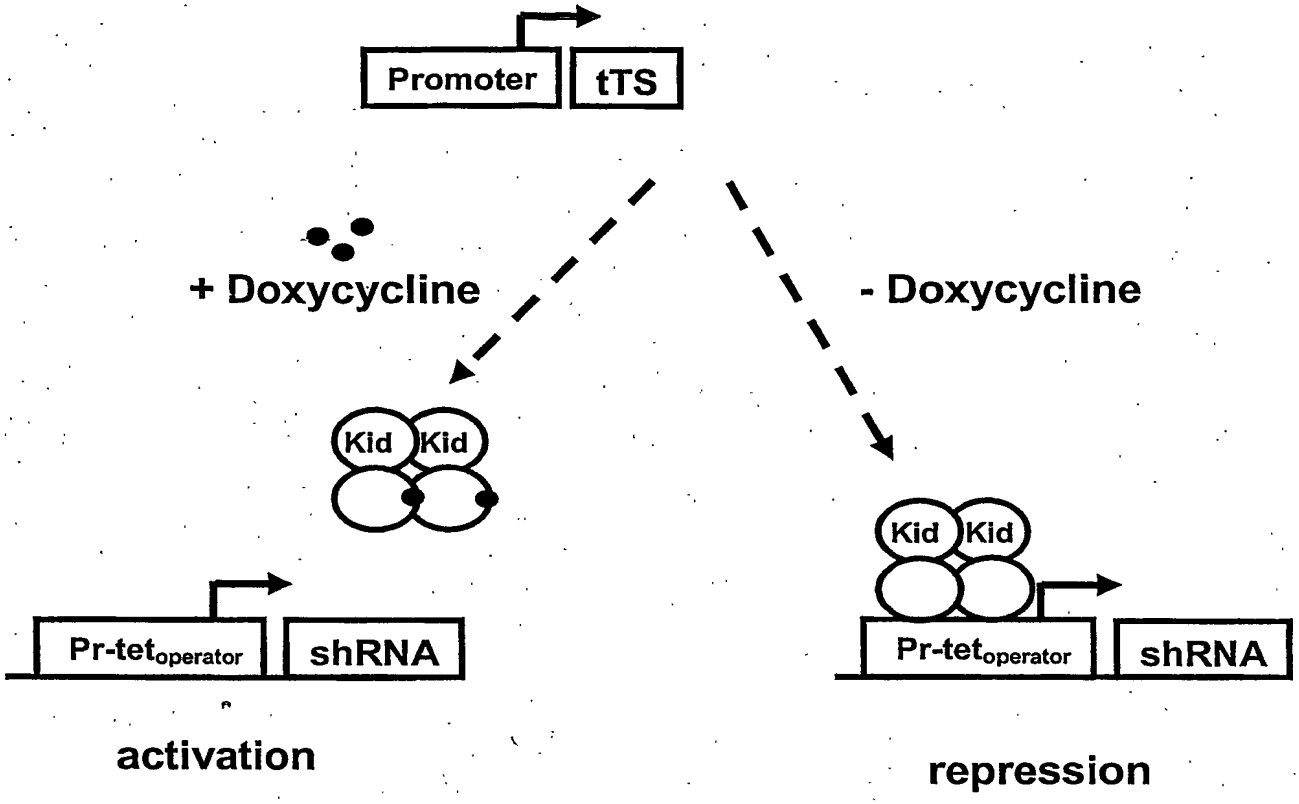
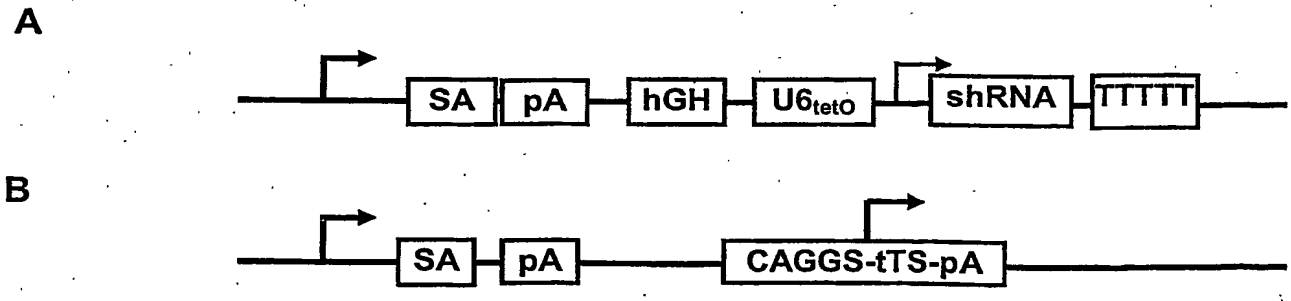

Promoter
tTS
+ Doxycycline
- Doxycycline
Kid
Kid
Kid
Kid
Pr-tet operator
shRNA
Pr-tet operator
shRNA
activation
repression

Fig. 3


A
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pA
hGH
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shRNA
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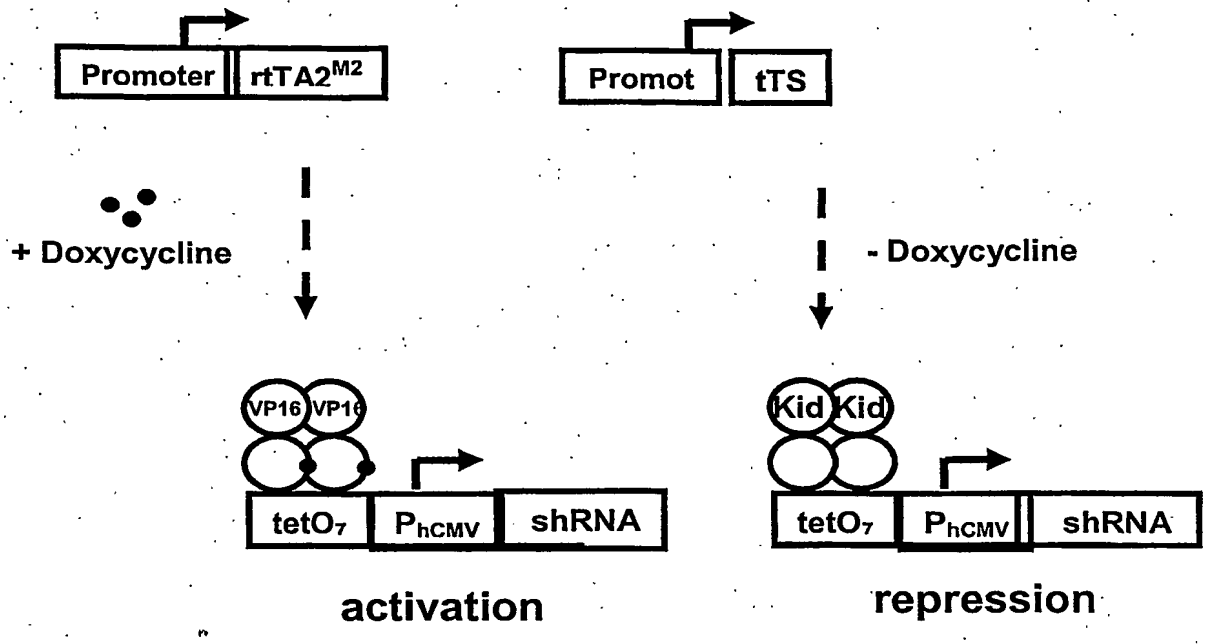
B
SA
pA
CAGGS-tTS-pA

Fig. 4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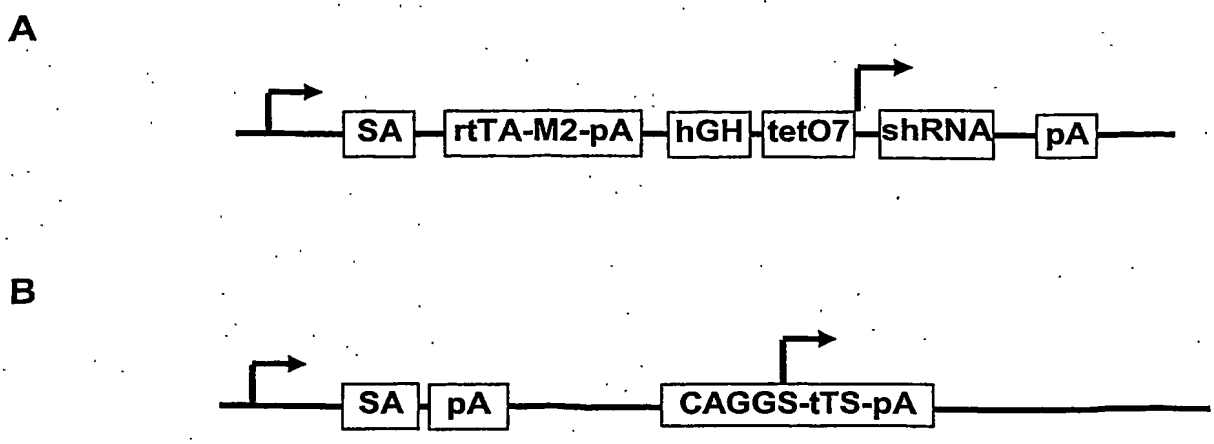
rtTA2M2
Promot
tTS
+ Doxycycline
- Doxycycline
VP16
VP16
Kid
Kid
tetO7
PhCMV
shRNA
tetO7
PhCMV
shRNA
activation
repression

Fig. 5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hGH
tetO7
shRNA
pA
B
SA
pA
CAGGS-tTS-pA

Fig. 6

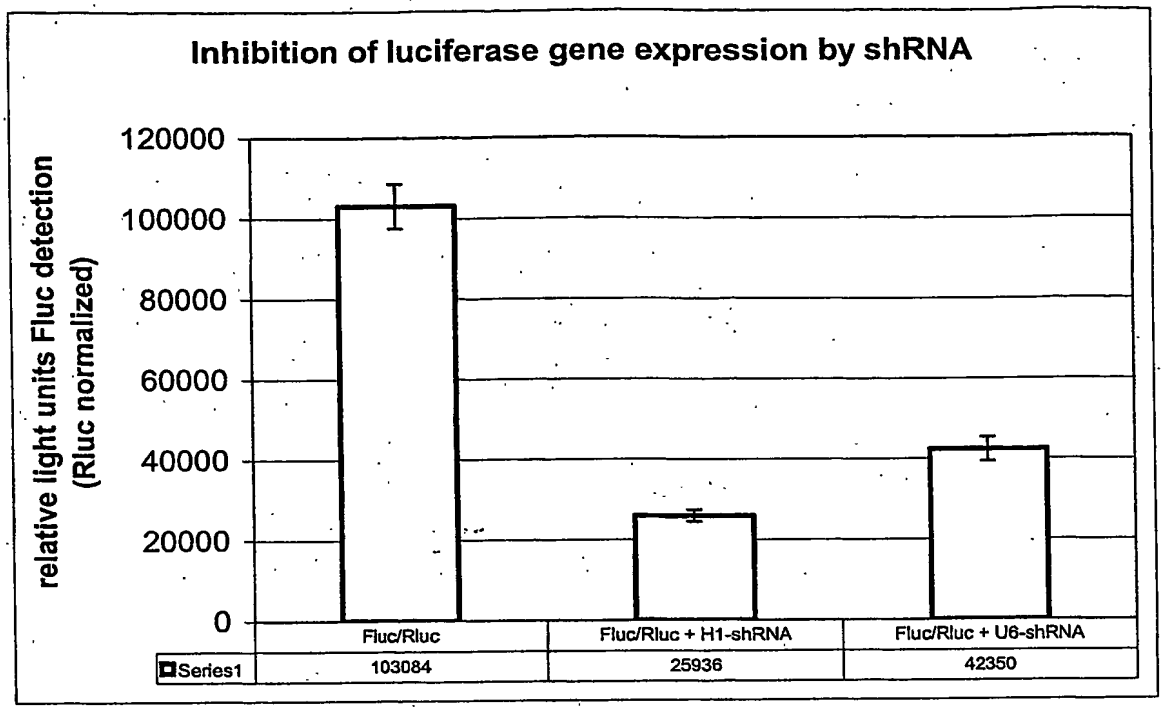


Fig.7

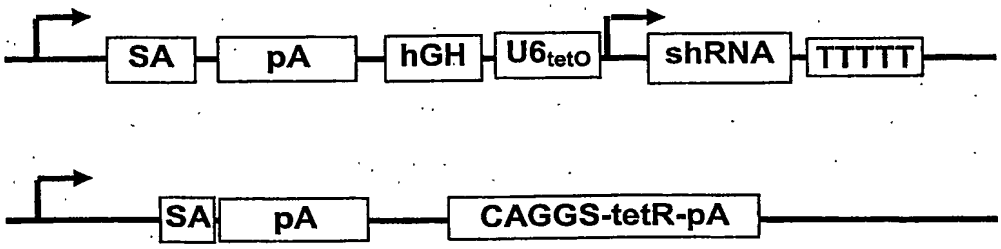


Fig.8

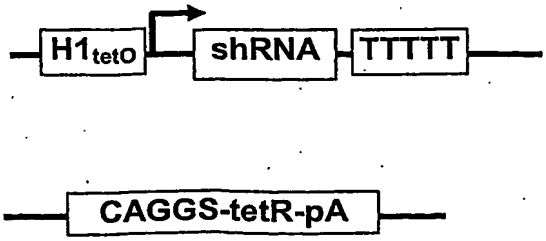


Fig.9

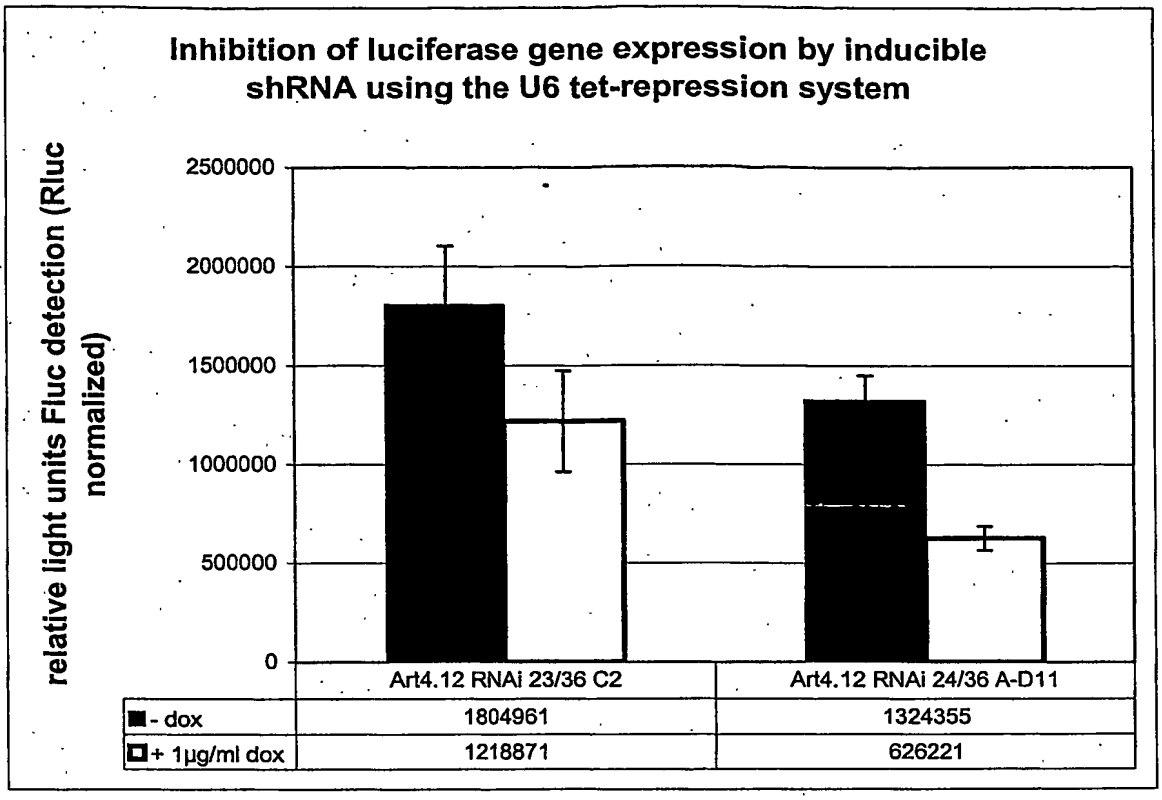

Inhibition of luciferase gene expression by inducible shRNA using the U6 tet-repression system
relative light units Fluc detection (Rluc normalized)
2500000
2000000
1500000
1000000
500000
0
Art4.12 RNAi 23/36 C2
Art4.12 RNAi 24/36 A-D11
- dox
1804961
1324355
+ 1µg/ml dox
1218871
626221

Fig.10

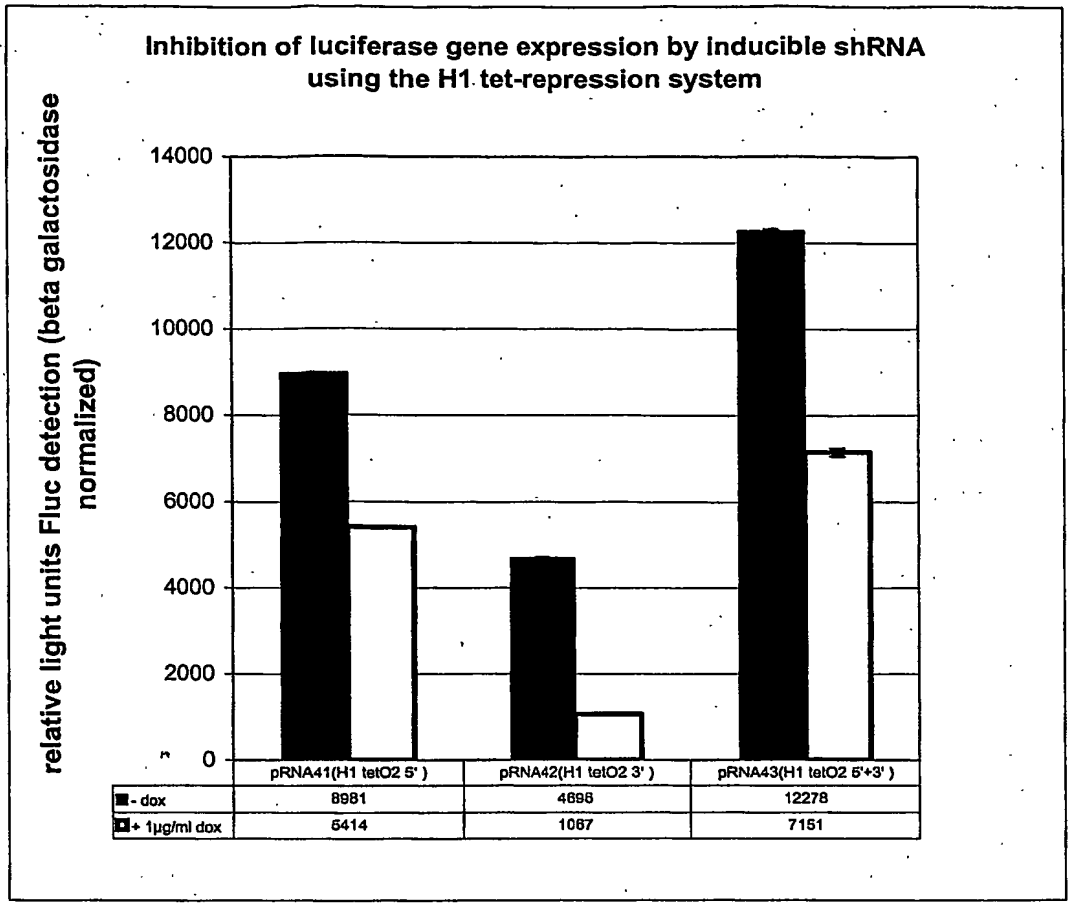

Inhibition of luciferase gene expression by inducible shRNA using the H1 tet-repression system
relative light units Fluc detection (beta galactosidase normalized)
14000
12000
10000
8000
6000
4000
2000
0
pRNA41(H1 tetO2 5')
pRNA42(H1 tetO2 3')
pRNA43(H1 tetO2 5'+3')
- dox
8981
4698
12278
+ 1µg/ml dox
5414
1067
7151

Fig.11

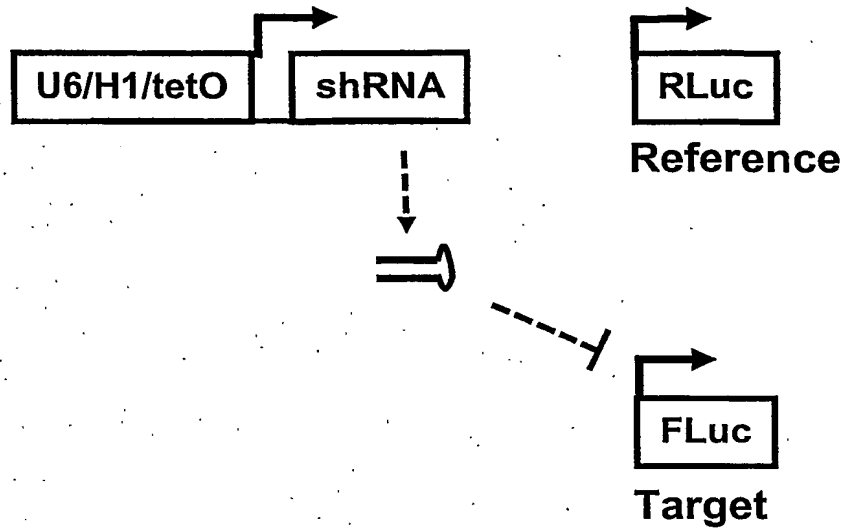
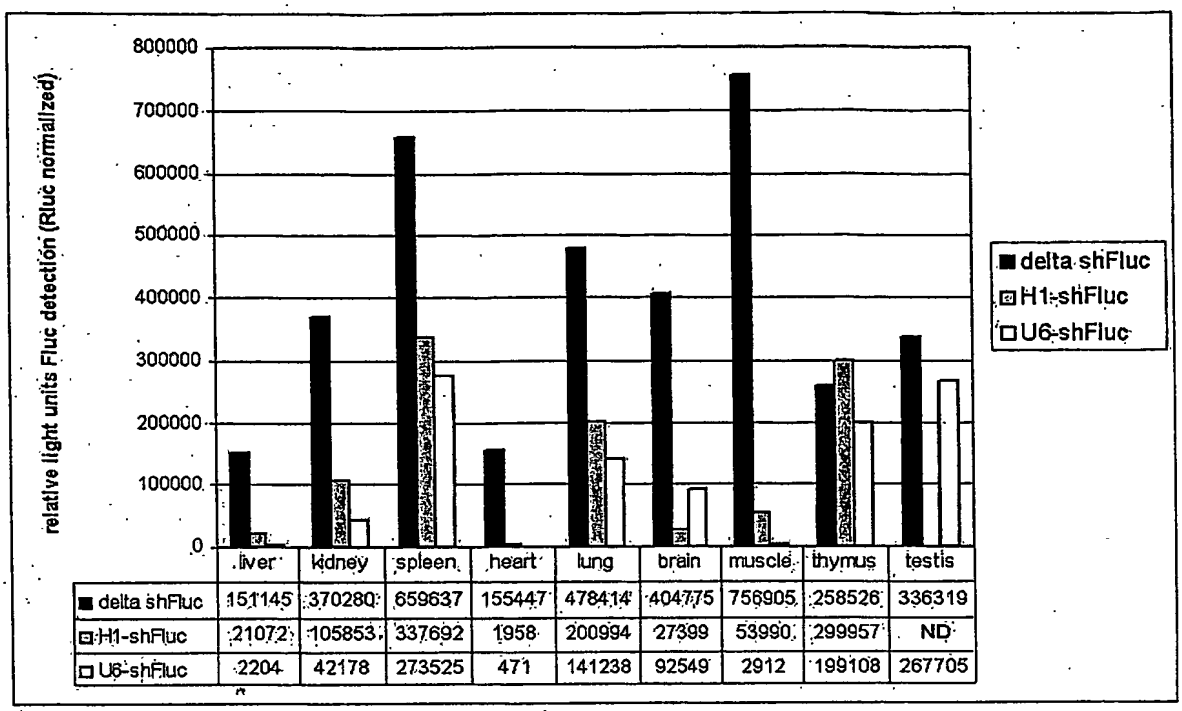


Fig.12

- 8 / 8-



	liver	kidney	spleen	heart	lung	brain	muscle	thymus	testis
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H1-shFluc	21072	105853	337692	1958	200994	27399	53990	299957	ND
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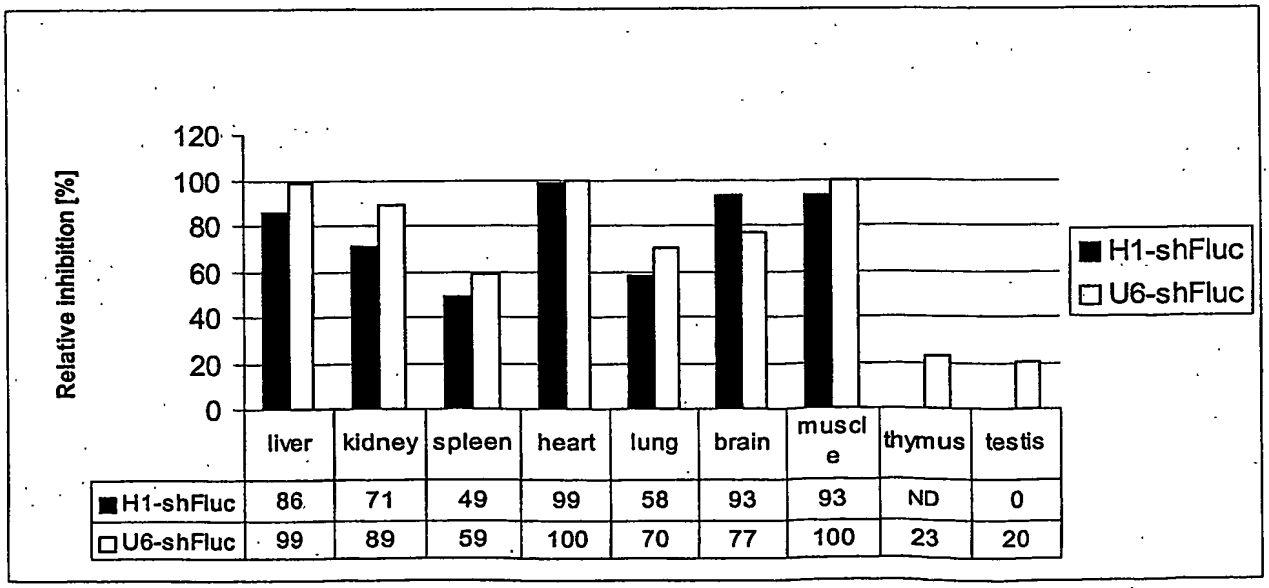
Fig. 13A



	liver	kidney	spleen	heart	lung	brain	muscle	thymus	testis
H1-shFluc	86	71	49	99	58	93	93	ND	0
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Fig.13B

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<210> 2
<211> 5409
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: sequence of homology region

<400> 2

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caggacatc 5409

<210> 3
<211> 4413
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Sequence of Fluc-hygro insert

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<210> 4
<211> 14947
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Targeting vector for Rosa26 locus with a Fluc-hygro insert

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<211> 4640
<212> DNA
<213> Artificial Sequence

<220>
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<211> 15174
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Targeting vector for Rosa26 locus with a Rluc-U6-shRNA neo insert

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<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Rluc tet02 insert

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<211> 3387
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: H1 tet5' shRNA vector

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<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: H1 tet3' shRNA
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<211> 3387
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: H1 tet5' + 3'
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<211> 7209
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:
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<211> 7014
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: GAGGS-Fluc

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(72) Inventors: SEIBLER, Jost; Leipziger Platz 13, 50733 Köln (DE). SCHWENK, Frieder; Kornradenweg 15, 50999 Köln (DE). KÜHN, Ralf; Hellenthaler Str. 22, 50937 Köln (DE). KÜTER-LUKS, Birgit; Maybachstr. 170, 50670 Köln (DE).

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(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW.

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Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2004/035782 A3

(54) Title: SIRNA MEDIATED GENE SILENCING IN TRANSGENIC ANIMALS

(57) Abstract: The present invention relates to a process that enables constitutive and inducible gene knock down in living organisms using a short hairpin RNA expression vector integrated into the genome of the organism.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 03/11233

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/11 C12N15/63 C12N5/10 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, MEDLINE, EMBASE, EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DEVROE E & SILVER P A: "Retrovirus-delivered siRNA" BMC TECHNOLOGY, BIOMED CENTRAL, LONDON, GB, 28 August 2002 (2002-08-28), pages 1-5, XP002225637 ISSN: 1472-6750 cited in the application the whole document	1,2,4-7, 11,13
Y		3,8-10, 12
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
25 March 2004	14/04/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mauhin, V

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 03/11233

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BRUMMELKAMP T R ET AL: "A system for stable expression of short interfering RNAs in mammalian cells" SCIENCE, AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE,, US, vol. 296, no. 5567, 2002, pages 550-553, XP002225638 ISSN: 0036-8075 cited in the application the whole document	1,2,4-6, 11-13
X	PAUL C P ET AL: "EFFECTIVE EXPRESSION OF SMALL INTERFERING RNA IN HUMAN CELLS" NATURE BIOTECHNOLOGY, NATURE PUBLISHING, US, vol. 20, no. 5, May 2002 (2002-05), pages 505-508, XP001121066 ISSN: 1087-0156 cited in the application figure 1 table 1 Experimental protocol: Materials	1,2,4-7, 11,13
X	KISTNER ET AL: "Doxycycline-mediated quantitative and tissue-specific control of gene expression in transgenic mice" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, US, vol. 93, no. 20, October 1996 (1996-10), pages 10933-10938, XP002110022 ISSN: 0027-8424 the whole document	1-13
Y	BRONSON SARAH K ET AL: "Single-copy transgenic mice with chosen-site integration" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 93, no. 17, 1996, pages 9067-9072, XP002270939 1996 ISSN: 0027-8424 Introduction last paragraph page 9072	3,8-10, 12

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 03/11233

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	HASUWA H ET AL: "Small interfering RNA and gene silencing in transgenic mice and rats" FEBS LETTERS, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL, vol. 532, no. 1-2, 4 December 2002 (2002-12-04), pages 227-230, XP004395375 ISSN: 0014-5793 cited in the application the whole document	1,2,4-8, 10-13
X,P	WO 03 057840 A (ALLELE BIOTECHNOLOGY & PHARMAC) 17 July 2003 (2003-07-17) paragraphs '0008!-'0014! paragraph '0021! paragraphs '0023!,'0024! paragraphs '0041!,'0042! paragraphs '0046!-'0048! paragraph '0050! paragraph '0063! paragraph '0065! paragraphs '0069!,'0070! paragraphs '0072!-'0076! paragraph '0079! paragraphs '0112!,'0113! paragraphs '0115!-'0117! paragraph '0129! paragraph '0138! paragraphs '0142!-'0144! paragraph '0155!	1,2,4-8, 10-14
X,P	KAWASAKI H ET AL: "Short hairpin type of dsRNAs that are controlled by tRNA Val promoter significantly induce RNAi-mediated gene silencing in the cytoplasm of human cells" NUCLEIC ACIDS RESEARCH, OXFORD UNIVERSITY PRESS, SURREY, GB, vol. 31, no. 2, January 2003 (2003-01), pages 700-707, XP002965487 ISSN: 0305-1048 the whole document	1,2,4-7, 11,13
X,P	MATSUKURA SHIRO ET AL: "Establishment of conditional vectors for hairpin siRNA knockdowns." NUCLEIC ACIDS RESEARCH. ENGLAND 1 AUG 2003, vol. 31, no. 15, 1 August 2003 (2003-08-01), pages (e77) 1-5, XP002270940 ISSN: 1362-4962 the whole document	1,2,4-6, 11,13

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 03/11233

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	VAN DE WETERING MARC ET AL: "Specific inhibition of gene expression using a stably integrated, inducible small-interfering-RNA vector." EMBO REPORTS, vol. 4, no. 6, June 2003 (2003-06), pages 609-615, XP002270941 ISSN: 1469-221X (ISSN print) the whole document	1,2,4-7, 11,13
A	MERLINO G T ET AL: "ACCURATE IN-VITRO TRANSCRIPTIONAL INITIATION OF THE CHICK ALPHA-2 TYPE I COLLAGEN GENE" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 256, no. 21, 1981, pages 11251-11258, XP002270942 ISSN: 0021-9258	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 03/11233

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-10 are partially directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 03/11233

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 03057840 A	17-07-2003	WO 03057840 A2	17-07-2003
		US 2003175772 A1	18-09-2003

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